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Anti-inflammatory Activty of *Oryza sativa* L. (Njavara)

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Abstract

Oryza sativa L. (Njavara), a widely used Ayurvedic system of medicine, especially in Panchakarma treatment is Kerala's own wonder rice (Poaceae). As there is no scientific evidence for the pharmacological properties of Njavara, the present study evaluated the anti-inflammatory activity of Njavara grain, in both *in vivo* and *in vitro* models. In the *in vivo* model of experiment, Njavara methanolic extract (NME) significantly inhibited paw edema in carrageenan induced rats. In the *in vitro* tests, the probable supporting mechanism by which NME mediates its effects on inflammatory conditions was studied on human peripheral blood mononuclear cells exposed to lipopolyscaccharide with increasing concentrations of NME substantially attenuated the increase in levels of various inflammatory enzymes like COX, LOX, NOS, MPO and oxidative stress induced by LPS in cell culture. Thus Njavara possess potent anti-inflammatory effect and can be used for the treatment of inflammatory disorders.

Key words: anti-inflammatory, Njavara methanolic extract, carrageenan induced inflammation, lipopolysaccharide, *Oryza sativa* L.

1. Introduction

Inflammation is a protective mechanism of the body against infectious agents and injury. Common symptoms are redness, swelling, heat, pain and deranged functions [1]. Chronic inflammation is a continuous inflammatory disease state driven by the development of an immune response to an endogenous antigen [2]. Chronic inflammation is the basis for rheumatoid arthritis [3] and cardiovascular disease [4].

Oxygen derived free radicals and their products are known to play an important role in the pathogenesis of chronic inflammatory disorders [5, 6]. Free radicals can directly or indirectly damage basic articular constituents and lead to the clinical expression of the inflammatory arthritis [7]. Oxidative stress is also considered to play a key role in the pathogenesis of atherosclerosis as evidenced by the increase in the oxidative stress with individual risk factors of atherosclerosis such as obesity, hypertension, hyperlipidemia, diabetes and smoking [8].

Cyclooxygenase (COX) and Lipoxygenase (LOX) enzymes have significant roles in inflammation [9, 10]. Prostaglandin E2 (PGE_2)

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is one of the major end-products of the cyclooxygenase-2 pathway, an enzyme that is an important mediator of inflammation [11]. PGE_2 is synthesized in substantial amounts at sites of inflammation where it acts as a potent vasodilator and sinergistically with other mediators such as histamine and bradykinin causes an increase in vascular permeability and edema [12]. Leukotrienes, the end products of lipoxygenase pathwayplay a major role in the inflammatory response injury; they have been implicated in the pathogenesis of inflammatory diseases, most notably asthma, psoriasis, arthritis and inflammatory bowel disease [13, 14].

Nitric oxide synthase (NOS) and myeloperoxidase (MPO) are considered as markers of endothelial dysfunction in rheumatoid arthritis (RA) and atherosclerosis [15, 16]. Nitric oxide contribute to tissue damage in inflammation [17] and may also trigger activation of various proteins that inturn activate the inflammatory response. MPO was proposed to have powerful prooxidant and proinflammatory activities and considered as a marker of neutrophil activity [18]. Anti-inflammatory drugs and agents reduce the inflammatory response by suppressing the pathway of production of the inflammatory enzymes and in turn block the initiation and progression of inflammation-associated diseases [19, 20].

Due to side effects of non steroidal anti-inflammatory drugs (NSAIDs), herbal therapies are well appreciated. *Oryza sativa* . commonly known as Njavara of the family Poaceae is a medicinal rice traditionally used in ayurveda. Kizhi and Njavara Theppu are two major treatments in Ayurveda in conditions of arthritis, paralysis, neurological complaints, degeneration of muscles, tuberculosis, for children with anemia, for women during lactation, in certain ulcers and skin diseases. As there is no scientific evidence for the pharmacological properties of Njavara, the present study evaluated the anti-inflammatory activity of Njavara.

2. Materials and methods

2.1. Chemicals

High quality analytical grade reagents were used for all experiments. RPMI 1640, Penicillin, Streptomycin, Gentamycin and Amphotericin and other were purchased from Sigma chemicals Co, USA and chemicals from SRL chemicals, Mumbai, India.

2.2. Plant material

Authentic Njavara grain samples of 'black glummed' variety were collected directly from the certified producer namely the Organic certified 'ECO FARM' karukamanikalam at Chittoor, Palakkad, Kerala. The identity of the black glummed Njavara sample was verified and confirmed by Dr.Maya C.Nair, Faculty (Sr.Grade Lecturer), Department of Botany, Government Victoria College, Palakkad - 678 001, Kerala, India, as identical with the specimen sample IC 539968 deposited at National Bureau of Plant Genetic Resources, (NBPGR), New Delhi, India, against collection (voucher) No.MS004/05.

2.3. Preparation of Njavara extracts

10g of the grain was immersed in 100 mL methanol and ethanol in separate conical flasks. Another 10g was boiled with water, filtered and immersed in 100 mL ethanol. Kept in a cold room for 48 hrs and filtered with cheese cloth to their respective extracts. A small volume of the extract was evapourated in a pre- weighed dish and quantitated the residue of the preparation.(Yield: methanolic extract-32 g/Kg grain; ethanolic extract - 60 g/Kg grain; ethanolic extract of boiled grain-80 g/Kg grain)

2.4. Animals

Female rats (Sprague Dawley strain) (120 - 200 g) were for the experiments. These animals we re bred and reared in the department animal house and maintained on normal laboratory diet (Gold Mohur rat feed, Hindustan Lever Ltd.). Water was provided ad libitum. The rats were housed in polypropylene cages in room with temperature maintained at 25 ± 1^0C and 12 hour light and dark cycle. Experimental procedures conducted on rats were reviewed and approved by the Animal Experiment Committee according to the Government of India accepted principles for lab animal use and care.

2.5. Experimental design

2.5.1. In vivo experiments

For the selection of Njavara extract with maximum anti-inflammatory activity, an *in vivo* experimental model like carrageenan induced inflammation was employed.

Animals were divided into six groups.

Group-I- Normal (received saline)

Group II- Control (subcutaneous administration of 0.1 mL 1% Carrageenan at the hind paw)

Group III- Carrageenan + Njavara Methanolic extract (NME)

Group IV- Carrageenan + Njavara Ethanolic extract (NEE)

Group V- Carrageenan + Boiled Njavara Ethanolic extract (BNEE)

Group VI- Carrageenan + Indomethacin (3 mg/kg)

Extracts (100 mg/Kg) and the known NSAID, Indomethacin were given orally 30 minutes before carrageenan administration. Paw volume was measured by plethysmograph [21].

2.5.2. In vitro experiments

For understanding the mechanism of anti-inflammatory action of Njavara extract, human peripheral blood mononuclear cells (hPBMCs) were used as the *in vitro* experimental model. Blood was collected from healthy individuals, cells were isolated by density gradient centrifugation using Histopaque 1077 and cultured in 35mm collagen coated culture plates using RPMI 1640. After giving 4 hours for attachment, the medium and unattached cells were removed and supplemented with fresh medium. The cells were maintained at 95% air and 5% CO_2 on a Sanyo CO_2 incubator maintained at 37^0C. The medium was removed and supplemented with fresh medium after 24 hours and subjected to further treatment.

Cells were treated as different groups; Group I- Normal; Group II-Lipopolysaccharide (LPS) control (1 μg/ml); Group III- LPS+NME (10 μg/ml); Group IV- LPS+NME (25 μg/ml); Group V- LPS+NME (50 μg/ml); Group VI- LPS+NME (100 μg/ml). NME was given 1hr prior to LPS priming and the duration was 24hrs.

For the biochemical evaluation of inflammatory enzymes like COX, LOX, MPO and NOS, the lysed cellular fraction by performing three freeze thaw cycles using liquid nitrogen was used as the enzyme source. Subsequently media were collected to measure the activity of superoxide dismutase (SOD) and level of malondialdehyde (MDA).

2.6. Biochemical examinations

Lipoxygenase assay was done by Axelrod *et al.* [22] and cyclooxygenase by thiobarbituric acid method [23]. MPO activity was measured as an index of inflammatory using a previously reported method [24]. Nitric oxide synthase was determined by the method described by Salter *et al* [25]. Superoxide dismutase activity was determined by the method described by Kakkar *et al* [26]. MDA was evaluated by the

thiobarbituric acid reacting substance (TBARS) method [27]. Briefly, MDA reacted with thiobarbituric acid in the acidic high temperature and formed a red-complex TBARS. The absorbance of TBARS was determined at 532 nm. Protein content was assayed by method of Lowry *et al* [28].

2.7. Statistical Analysis

The results were analyzed using a statistical program SPSS / PC +, version 11.0 (SPSS Inc., Chicago, IL, USA). One-way ANNOVA was employed for comparison test of significant differences among groups were determined. Pair fed comparisons between the groups was made by Duncan's multiple range test. $P< 0.05$ was considered significant.

3. Results & Discussion

Acute inflammation is a part of defence response but chronic inflammation is found to mediate a wide variety of diseases like cancer, diabetes, arthritis, Alzheimer's disease, pulmonary disease and auto immune diseases [29]. Carrageenan induced paw edema is a as a working model of in the search for new anti-inflammatory drug [30]. The anti-inflammatory activity of different extracts of Njavara grain were evaluated by carrageenan induced rat paw edema method. A considerable increase in the percentage of paw edema inhibition was observed in all groups on 3rd, 5th and 24th hour of carrageenan induction. The edema was significantly ($P\leq0.05$) reduced or maximum inhibition was observed in Njavara methanolic extract (NME) treated group than the groups treated with ethanolic and boiled ethanolic extracts. Results are shown in table A . The effect of NME was comparable with the commonly used NSAID, Indomethacin and the effect of NME lasted for over 24 hours

Table A: Effect of Njavara extracts on carrageenan induced inflammation model

Group	Standard error mean (SEM)		
	3rd hour	5th hour	24th hour
NME	2.02	2.3	2.59
NEE	1.44	1.85	1.85
BNEE	0.479	0.721	1.32
Indomethacin	1.15	1.96	2.3

Table B: Effect of NME on cyclooxygenase activity in LPS induced hPBMCs

Group	Standard error mean (SEM)
Normal	0.004
LPS treated	0.003
10 µg/ml	0.003
25 µg/ml	0.003
50 µg/ml	0.004
100 µg/ml	0.004

Table C: Effect of NME on lipoxygenase activity in LPS induced hPBMCs

Group	Standard error mean (SEM)	
	5-LOX	12-LOX
Normal	0.004	0.003
LPS treated	0.003	0.003
10 µ/ml	0.003	0.003
25 µ/ml	0.003	0.003
50 µ/ml	0.004	0.004
100 µ/ml	0.003	0.004

Table D: Effect of NME on myeloperoxidase activity in LPS induced hPBMCs

Group	Standard error mean (SEM)
Normal	6.01
LPS treated	6.01
10 µg/ml	6.6
25 µg/ml	1.94
50 µg/ml	1.2
100 µg/ml	7.05

Table E: Effect of NME on nitricoxide synthase activity in LPS induced hPBMCs

Group	Standard error mean (SEM)
Normal	0.15
LPS treated	0.36
10 μg/ml	0.45
25 μg/ml	0.39
50 μg/ml	0.33
100 μg/ml	0.15

Table 1: Effect of NME on oxidation status in monocyte culture.

Group	SOD(unit$/mg protein)	MDA(nmol/mg protein)
Normal	39.78±0.72	15.31±0.30
LPS	12.86±0.44*	97.50±0.28*
LPS+10 μg/ml NME	14.58±0.63#	47.38±0.32#
LPS+25 μg/ml NME	18.88±0.79#	31.46±0.46#
LPS+50 μg/ml NME	34.86±0.82#	28.71±0.77#
LPS+10 μg/ml NME	36.82±0.76#	16.43±0.33#

Effect of NME on oxidative status in LPS induced hPBMCs
The cells were pre-treated with increasing doses of extract prior to LPS challenge. Values are expressed as average of 6 samples ±SEM in each group. * Significantly different from normal control ($p < 0.05$). # Significantly different from LPS control ($p < 0.05$). $SOD-U=enzyme concentration required to inhibit chromogen production by 50% in 1min.

indicating its anti-inflamamtory potential. So, further studies were carried out on methanolic extract of Njavara.

Inflammatory processes are recognized to play a central role in the pathogenesis of atherosclerosis and its complications. Considering the critical role of monocytes / macrophages in the inflammatory process and atherosclerosis [31], an *in vitro* cell-based system using hPBMCs after LPS stimulation was used to test the mechanism of anti-inflammatory effects of NME. In response to LPS, monocytes produce both proinflammatory mediators like arachidonic acid derivatives regulatory proteins that counteract the inflammation and oxidative stress [32]. NME was found to be inhibiting both the cyclooxygenase and pathways (table B and C) of arachidonic acid metabolism shows the anti-inflammatory potency of the drug.

MPO is widely used as a biomarker for neutrophil associated inflammation and is proposed to reflect neutrophil activity. Activated neutrophils infiltrating the sites of inflammation are an important source of oxygen derived free radicals and proinflammatory mediators [33, 34]. NME was found to be dose dependently restoring normal neutrophil activity by significantly ($P \leq 0.05$) decreasing MPO activity in LPS induced monocytes (table D). This suggests that prevention of associated inflammation is another mechanism by which NME achieves its anti-inflammatory effect.

Another mediator reported to play an important role in inflammation is NO [17]. Previous

studies have demonstrated that the suppression of biological activities of iNOS by neutralizing antibodies, selective inhibitors or gene targeting have led to a dramatic improvement in the local inflammation and progression of RA [35] atherosclerosis [36]. NME significantly inhibited total NOS activity and there by blocked the inflammatory changes exerted by nitric oxide, compared to LPS induced inflammation control (table E). This cleared the ability of NME in blocking the activation of inducible enzymes of inflammation which regulates chronic changes in pathogenesis.

Decreased antioxidant status or the stressful oxidative condition leads to the of chronic inflammatory disorders [5, 6]. Previous data have showed that the level of MDA was decreased and SOD was increased by antioxidant and anti-inflammatory agents [37]. In present study, compared with the normal group, the level of SOD was decreased, while MDA contents were increased remarkably in the LPS control group (Table 1). This stressful condition was reversed by treatment with NME. NME provided protective effects in LPS primed inflammation probably by the radical scavenging and antioxidant properties in a dose dependent manner. This may be an important and underlying mechanism of NME in protection against inflammation.

Methanolic extract of Njavara exerted anti-inflammatory activity by preventing the paw edema formation in carrageenan induced rats. Studies conducted in hPBMCs showed the dose dependent anti-inflammatory activity of NME, as evidenced by normalizing the upregulated activity of various inflammatory enzymes like COX, 5-LOX, 12-LOX, NOS and MPO and alleviating the oxidative stress. Results obtained from this study indicate that Njavara grains may have therapeutic applications for inflammatory disorders or preventive activity against inflammation. Further studies are going on to isolate the active component and to understand the molecular mechanism of its action.

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